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(57) Abstract

Peptides comprising a sequence the same as or immunologically equivalent to a linear epitope of the carboxy terminal region of *Mycobacteria tuberculosis* heat shock proteins such as Hsp71 useful in the diagnosis of paucibacillary tuberculosis (TB). Methods of diagnosis of TB using such peptides and test kits including such peptides are also provided.

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PEPTIDE FRAGMENTS OF HSP71 OF M. TUBERCULOSIS AND THEIR USE IN DIAGNOSIS OF TUBERCULOSIS

The present invention relates to peptides which are useful in diagnosing paucibacillary mycobacterial infections.

The paucibacillary stage of tuberculosis is very hard to detect or diagnose due to the effective absence of clinical signs and symptoms and the lack of detectable pathogen-originating antigens. Diagnosis in the multibacillary stage is relatively easy, for instance using well known smear test. Diagnosis at the paucibacillary (or smear-negative) stage is important in order that appropriate treatment and containment measures can be implemented to avoid transmission to persons at the greatest risk infection. To date efforts to improve the diagnosis of paucibacillary TB have been unsuccessful.

The present inventors have now identified certain peptides which may be used in detecting paucibacillary TB and other mycobacterial diseases. The peptides are related to the carboxy terminal of the stress protein Hsp71 of Mycobacterium tuberculosis and corresponding heat shock proteins of other mycobacterial pathogens.

Without wishing to be bound by this theory the

present inventors consider that the carboxy terminal regions of Hsp71 and corresponding mycobacterial heat shock proteins contain species-specific determinants having linear epitopes. These linear epitopes generate an antibody response during the paucibacillary stage of infection and peptides having corresponding sequences can be used to detect the antibodies in the serum or other body fluids of the patient.

Peptides of the invention may be used for detecting or diagnosing a variety of paucibacillary mycobacterial diseases especially, but not limited to, tuberculosis of humans and wild or domestic mammals such as cattle, donkeys, deer and badgers. The peptides may also be used in detecting or diagnosing multibacillary mycobacterial infections.

The present invention therefore provides a peptide comprising a sequence the same as or immunologically equivalent to a linear epitope of the carboxy terminal region of M. tuberculosis Hsp71 or a corresponding heat shock protein of any other mycobacterial pathogen.

Preferably the peptide comprises a sequence the same as or immunologically equivalent to a linear epitope of the carboxy terminal of M. tuberculosis Hsp71.

The invention will be illustrated with reference to the figures of the accompanying drawings in

which:-

Figure 1 shows the aligned carboxy terminal region sequences of M. tuberculosis Hsp71 and corresponding heat shock proteins using the internationally-accepted 1-letter code for amino acid residues. Hu=Human hsp70-1, Ec=Escherichia coli dnaK, Lp=Mycobacterium leprae Hsp71, Tb=Mycobacterium tuberculosis Hsp71 .=sequences identical with M.tuberculosis. -=gaps.

Figure 2 shows an analysis of antigens by SDS PAGE and Western blots. Ec=extract from E coli a=coomassie blue stain; b=Western blots developed with tuberculosis patient's serum.

Figure 3 gives individual serum antibody titers to mycobacterial hsp70 (M-hsp70) and human hsp70(H-hsp70 in groups of patients and controls. Groups:active TB(A); "self-healed" inactive TB(B); past treated inactive TB (C); lung fibrosis (D); diseases other than tuberculosis (E); healthy BCG-vaccinated individuals (F) (see also table 1). Group means are indicated by the horizontal bars. Boxed symbols in group B: Patients given

chemoprophylaxis due to severity of chest X-ray and clinical evaluation.

Figure 4 gives antibody levels in active tuberculosis divided according to sputum bacillary status. S=smear microscopy; C=culture of M tuberculosis. Group means are indicated by horizontal bars.

Figure 5 gives the ratios between anti-mycobacterial and anti-human hsp70 antibody levels. Groups of patients:see legend to table 1.

Figure 6 Shows antibody binding to octamer peptides derived from the carboxy-terminal sequence of the hsp71 protein M.tuberculosis. Serum from a patient with sputum smear-negative pulmonary tuberculosis, diluted 1:400 was reacted with solid-phase bound peptides using the ELISA method.

Figure 7 Shows localization of epitopes within the carboxy-terminal hsp71 sequence. Mean absorbance values from eight adjacent octamer peptides each containing the individual amino acid residues listed on the horizontal scale.

Sera tested at 1:400 dilution were collected from patients with smear-negative pulmonary tuberculosis (PT, see binding to individual peptides in figure 6), lung disease caused by M.avium(AM) and lepromatous leprosy (LL). A-D=localisation of epitope core hexamers.

Figure 8 Shows semi-quantitive evalution of antibodies to four (A-D) epitopes. Sera diluted 1:400 were collected from patients with smear-positive (full circle) or smear-negative (shaded circle) tuberculosis and from non-tuberculosis controls (open circles) represented by one serum each from lepromatous leprosy, lung mycobacteriosis, Crohn's disease and a healthy subject. All sera were pre-selected to contain high antibody levels to the whole hsp71 antigen. Absorbances (vertical scale) are mean values from adjacent peptides corresponding to each of the four epitopes (bottom horizontal scale).

The sequences of the carboxy terminal regions of M. tuberculosis Hsp71 and the corresponding human Hsp70 and Escherichia coli dnaK molecules and the Hsp71 of M. leprae are set out in Figure 1. Four linear epitopes within the Hsp71 of M. tuberculosis are indicated by the

core sequences A to D underlined in Figure 1. It is presently preferred that the peptide of the invention comprises at least one of these linear epitope core sequences or an immunological equivalent thereof.

Core sequences A and C, especially the latter, which appear to be more highly specific to M. tuberculosis, are preferred for selective diagnosis of paucibacillary TB in humans. Core sequences B and D appear to be more immunogenic than sequences A and C but have some cross reaction between M. tuberculosis and M. leprae; these core sequences are therefore preferred for more highly sensitive diagnosis of paucibacillary TB where infection by M. leprae can be ruled out on other grounds.

The linear epitopes may be longer or shorter than the 6-residue core sequences given in Figure 1 but are unlikely to be less than five residues in length. Accordingly, preferred peptides of the invention comprise at least any five and preferably all six amino acid residues of one of the core sequences indicated in Figure 1. More preferred peptides are octamers containing five or preferably all six of the amino acid residues of one of the core sequences and especially preferred peptides are octamers having the whole core sequence and two other residues corresponding to the two flanking residues at either the N- or C- flanks or one residue at each flank in

the M. tuberculosis Hsp71 sequence given in Figure 1.

Other preferred peptides of the invention contain a sequence immunologically equivalent to one of the aforementioned linear epitopes, i.e. peptides which cross-react with antibodies detected by peptides containing a linear epitope sequence; more preferably such immunological equivalents contain sequences which cross-react with one of the aforementioned core sequences.

The peptides of the invention may also contain sequences unrelated to Hsp71 or corresponding molecules, at either or both flanks of the core sequence or linear epitope sequence. In a particularly preferred embodiment the peptide comprises a branched polylysine bearing multiple copies of a linear epitope sequence or immunological equivalent thereof.

Peptides of the present invention may be produced by well known peptide synthetic techniques using solid or liquid-phase reactions to assemble the peptide one amino acid residue at a time or by covalently binding preformed sub-sequences, e.g. by linking linear epitope sequences or immunological equivalents thereof to a branched polylysine or other branched structure. Branched polylysines and other suitable branched structures are commercially available or described in the literature and linking of peptides to these may be achieved by conventional techniques.

Techniques for identifying linear epitopes and immunological equivalents thereof are readily available to those skilled in the art.

The peptides of the invention may be used in any conventional immunodiagnostic test technique for detecting antibodies in a body fluid such as blood or serum.

The invention further provides a diagnostic test process comprising contacting a peptide as hereinbefore defined with a body fluid sample taken from an individual suspected of having a mycobacterial infection. The peptide may be contacted in the liquid phase or the solid phase, in which case the peptide will be bound to a solid carrier such as the test vessel, for instance a microtitre plate, or latex beads. Binding of antibody from the sample to the peptide may be detected by any conventional technique.

For use in certain diagnostic procedures it may be advantageous to bind the peptide to a detectable label such as an enzyme label, a chromophore, a fluorophore or a radio-isotope or to a solid support. Binding of the peptide to a label or solid supports may be effected by conventional techniques. Peptides bound to such labels and solid supports form a further aspect of the invention.

Although it is presently envisaged that the peptides of the invention will be used in <u>in vitro</u>

diagnostic procedures, it is possible that in vivo techniques will be used. Accordingly, the invention further provides a peptide as hereinbefore defined, optionally bound to a label or solid support, for use in a diagnostic process practised on the human or animal body.

The invention further provides a process for producing a peptide as hereinbefore defined; a process for linking a peptide as hereinbefore defined to a detectable label or solid support; and tests kits containing the peptide optionally bearing a label or linked to a solid support and optionally also comprising buffer, positive or negative control materials, labelling reagents, reagents for detecting a label and other conventional components.

The invention will now be illustrated by the following Examples:-

EXAMPLE 1

INTRODUCTION

In the past, hemagglutinating antibodies to tuberculin PPD were demonstrated in the serum of patients with active tuberculosis (1,2,3). However, in order to achieve satisfactory specificity and sensitivity of serology, antigenic epitopes with both species-specificity and strong immunogenicity would be required. Recent advances in the molecular definition of mycobacterial antigens has been exploring possibilities for developing a clinically useful test for the serodiagnosis of tuberculosis. In smear-positive (multibacillary) tuberculosis (TB), this requirement is best met by the 38 kDa protein antigen (reviewed in 4). However, in the case of smear-negative (paucibacillary) disease where laboratory diagnostic help is most required, the search for an "ideal" overlap in specificity and immunogenicity within a single epitope has not as yet been forthcoming.

Antibody levels to the 38kDa protein which represents the most specific and immunogenic antigen in multibacillary tuberculosis have been found much lower in smear-negative pulmonary tuberculosis (5,6); nevertheless, useful diagnostic discrimination of paucibacillary patients with this antigen was achieved using a more sensitive modification of the competition test (7). Of the other candidate antigens, about one half of smear-negative patients had a specific rise of antibodies against the 19kDa and to a lesser extent to the 14 kDa antigens (6), the

latter being compromised in its discriminatory potential by raised antibody levels in exposed healthy subjects (8). Consequently a combination of tests also involving antibody levels to the M tuberculosis-specific TB78-epitope of the hsp65 protein and lipoarabinomannan have been advocated (9).

In order to expand further the serological evaluation of mycobacterial antigens of defined structure, we examined in this paper antibody levels in patients and controls in respect of the hsp70 antigen. This protein, originally identified by monoclonal antibodies (10) has been sequenced by cloning its gene from the DNA of M. leprae and M tuberculosis (11,12). It belongs to the family of heat shock proteins with broadly conserved structure between prokaryotes and eukaryotes. At this is a strong structural basis for autoimmunization, it was of interest to compare serum antibody levels to both mycobacterial and human hsp 70 molecules in groups of patients and controls.

MATERIALS AND METHODS

Serum samples. All 198 tested sera were obtained from patients at the Northwick Park Hospital. (Informed consent was obtained from all patients and controls whose blood was sampled). On the basis of clinical diagnosis, they were classified to groups listed in table 1. Patients with active tuberculosis (group A:90 total) were further classified in respect of pulmonary localization, sputum smear microscopy and bacteriological culture. The clinical parameters of patients with extrapulmonary disease have

previously been described in greater detail (7). Patients with "inactive tuberculosis" (36 total) were represented by patients with radiological evidence of old pulmonary tuberculosis without history of drug treatment (group B "self-healed"), and those with a history of drug treatment (group C "past-treated"). Groups without active or past TB were represented by patients with clinical and radiologically demonstrable lung fibrosis (group D) and by patients with other diseases such as pneumonia, sarcoidosis, bronchiactasis, carcinoma of the lung, viral infections, liver diseases or heart failure (group E). Healthy subjects were age matched adult BCG-vaccinated individuals (group F).

Preparation of antigens: The gene coding the 71kDa protein of M tuberculosis inserted into the PUC8 multicopy plasmid was expressed in TG1 cells and purified by ATP-agarose chromatography (13). The material used in the study (M-hsp70) was prepared and kindly supplied from the WHO IMMTUB/IMMLEP Bank by Dr. Jan van Embden (Bilthoven, The Netherlands).

Recombinant human hsp70 protein was prepared from the stress-inducible hsp70 gene (14) kindly provided by Dr Rick Morimoto, Northwestern University, Evanston, IL., USA. It was subcloned in plasmid PET-3a (15) and expressed in E Coli using bacteriophage T_7 RNA polymerase system for selective high level expression (16). Hsp70 protein was purified from the crude protein lysates (Jindal and Young, in preparation).

The purity of both recombinant hsp70 antigens was

examined by SDS PAGE and Western blot analysis (figure 2). The results showed a single major band of 70 kDa for both the mycobacterial and human recombinant antigens. Only a few minor contaminating bands of lower molecular weight were detected in the H-hsp70 preparation with the human serum which reacted with several proteins in the crude E coli extract.

Table 1. Clinical Classification Of Patients

Group*	Clinical Diagnosis		Bacterial Status S^+C^+	S^-C^+	S^-C^-
A	Active TB	Pulmonary	30°	14	9
		Extrapulmonary	8	9	20
B	Inactive TB: self-healed		0	0	21
C		past-treated	0	0	15
D	Lung fibrosis		0	0	5
E	Other diseases		0	0	31
F	Healthy subjects		0	0	20

* - Corresponding with designations in figure 12

° - Number of patients

S = smear microscopy (+ or -)

C = culture of *M.tuberculosis* (+ or -)

Enzyme Linked Immunoassay (ELISA). Polyvinyl microtitre plates (Nunc-Immuno plate MaxiSorp F96) were incubated with 50μl of either M-hsp70 or H-hsp70 at concentration of 1 μg/ml or 2μg/ml respectively at 4°C overnight. After washing once with phosphate-buffered saline containing 0.05% (w/v) "Tween 20" (PBST), the wells were incubated with 200μl of 2% dried milk (w/v) in PBST (PBSTM) at 37°C for 1 hr. Four fourfold dilutions of human sera (1/50-1/3200) using 5°μl volume were added to duplicate wells and incubated at 37°C for 2 h. After 5 washings with PBST, 50μl of affinity purified goat antihuman IgG peroxidase conjugate (diluted 1/1000) was added to each well and the plates were further incubated for 1 h. After washing with PBST, 75μl tetramethyl benzidine plus hydrogen peroxide in citrate buffer at pH 5.1 was added to each well; the reaction was stopped in 10 min. time with 25μl per well 0.5 mol/L sulphuric acid. The absorbance at 450 nm was read in the "Titertek Multiskan II" spectrophotometer. Antibody titers were expressed as the dilution of serum giving 30% of the plateau binding value of a standard positive serum (ABT_{30}).

Statistical analysis: Means and standard deviations of the $\log_{10}$ values of antibody titers were calculated for each group of patients and controls. Groups were compared with each other by the student t-test for unpaired values. The proportion of patients with increased antibody levels in groups A-D was calculated with 98% specificity of the cut-off limit in comparison with values in group E.

RESULTS

Patients with diseases other than TB (group E) had antibody levels to both M-hsp70 and H-hsp70 significantly ($P < .001$) elevated above those found in healthy individuals (group F) (Figure 3). Therefore antibody levels in group E rather than those of the healthy controls (group F) were used as the "baseline" for evaluating the extent and significance of rise in antibody levels in groups with either active or inactive TB.

Patients with active tuberculosis (group A, Figure 3) had IgG antibody titers to M-hsp70 (mean $ABT_{30}3.2\pm0.4$) significantly higher ($P < .001$) than that of non-TB patients (group E) (mean ABT_{30} 2.3 ± 0.4). When taking the anti-M-hsp70 $\log_{10}$ titer 2.75 as the "cut-off" value 78% out of the 70 tested patients with active TB and significantly raised antibody levels. Corresponding evaluation of anti-H-hsp70 antibody levels using the $\log_{10}$ titer 2.5 as the discriminatory cut-off value in respect of group E, produced a somewhat lower, 50% positivity rate in patients with active TB.

Analysis of titers in groups of patients with "inactive tuberculosis" in comparison with group E deomonstrated that antibody levels have been increased to a significantly greater extent in self-healed (group B:80% for M-hsp70 and 67% for H-hsp70) than in the past treated (group C:53% for M-hsp70 and 47% for M-hsp70) individuals. In view of the large individual variations, we examined the possible association of antibody levels with several clinical

criteria such as age, race, duration of illness, chest X-ray, weight loss, fever and history of tuberculosis. However, none of these parameters were significantly correlated with antibody levels.

Interestingly, the clinical evaluation revealed that both anti-M and anti-H hsp70 titers were the highest in five patients of group B (boxed in figure 3) who were on basis of prior independent clinical and radiographic evaluation considered to be at risk of relapse and were therefore put on "secondary" rifampicin/isoniazid chemoprophylaxis. However, irrespective of the tuberculosis process, uniformly high antibody levels were also observed in all five tested patients with lung fibrosis (group D). Since most of the non-tuberculosis pulmonary and extrapulmonary diseases had "chronic inflammation" but did not have raised antibody levels, it would seem that anti-hsp70 antibodies are not stimulated merely by inflammation but rather represent a marker of the next stage, ensuing fibrosis.

It was of further interest to break down the antibody levels within the group with active TB (column A in figure 3) in relation to bacterial smear and culture positivity. The values expressed in Figure 4 showed no significant differences between the multibacillary (S^+C^+), intermediary (S^-C^+) and paucibacillary (S^-C^-) sub-groups in respect of either anti-M or anti-H- hsp70 antibody titers.

Comparison of antibody titers to M- and H- hsp70 antigen in individual patients showed highly significant correlation coefficients for all tested groups (mean R value

= 0.66). Nevertheless, it was noted that patients with active TB, had a relatively high ratio of titres to mycobacterial compared to human hsp70 (M/H ratio 4.2). The form of tuberculosis of the 11 patients with > 10 M/H ratio was pulmonary (3x), lymph node (4x), bone & skin (3x) and pericardial (1x). In contrast, a higher proportion of anti-human hsp70 titres (M/H ratio = 1.8) was found in patients with lung fibrosis and other non-TB diseases (figure 5).

DISCUSSION

Advances in the structural definition of mycobacterial antigens enabled a systematic study of specificity of the immune repertoire (4). Since, the relationships between the structure of antigenic molecules and their immune functions following infection with pathogenic mycobacteria are being established. The antibody repertoire of mice has been found to be distinctly more selective during infection with M. tuberculosis than following artificial immunization procedures (17). Hence, potential serodiagnostic antigens with high immunogenicity and specifically for the infecting pathogen need to be selected on the basis of detailed knowledge of the human immune system to pathogenic mycobacterial infection. The hsp70 protein, chosen for the present study, represents a well known major stress protein which is highly inducible under stress conditions (e.g. heat shock) and is one of the HLA linked hsp70 genes (18). Proteins of this family "chaperone" the folding and transport of intracellular

polypeptides. The 71kDa protein of M tuberculosis has biochemical and functional features consistent with other members of the hsp family (13) and has been found in supernatants from short-term culture of tubercle bacilli (19). The extracellular location may enable contact of the molecule in its native conformation with B cells which is a requirement for effective stimulation of antibody formation.

Despite the greatly conserved amino acid sequence composition of hsp70, it is of interest that existing monoclonal antibodies have been found restricted in binding mainly to pathogenic mycobacteria of the M. tuberculosis complex. M. leprae and M. scrofulaceum (10). Following infection with Schistosoma mansoni, antibodies also exhibited only limited cross-reactivity in respect of species specific epitopes of hsp70 and arose early in the course of infection (20,21). However, sera from humans infected with malaria contained autoantibodies which reacted with human hsp70 (22). Increased antibody levels to mycobacterial hsp70 have been found in sera of patients with active pulmonary tuberculosis (23, 24), but these studies did not show individual values and did not evaluate antibody levels in relation to the stage of disease.

The results in this study demonstrated significant elevation of anti-hsp 70 antibody levels in the majority (78%) of patients with active pulmonary TB. Furthermore, about 40% (based on the ratio between titres) of the antibodies appeared to be cross-reactive with the human hsp70 molecule. Despite its highly conserved aminoacid structure, the stimulation of anti-hsp70 antibodies appears

to be strongly associated with the tuberculosis infection since patients with a broad range of other diseases (excluding tuberculosis) had much less raised antibodies that TB patients, when compared with healthy controls. In this respect the anti-hsp70 response follows a quite different pattern than that to hsp65 which showed a very broad range of titres in non-TB controls which prevented any meaningful discrimination from tuberculosis patients (6). However, the persistence of raised hsp70 antibody levels for several years following active tuberculosis, which is known to apply also to other antigenic specificities, represents a caveat for diagnostic consideration.

The most striking finding has been the lack of any difference in the serological response to hsp70 between multibacillary and paucibacillary cases of tuberculosis. This finding is in contrast with the much more pronounced response of smear-positive TB patients to the previously examined M. tuberculosis specific epitopes (5,6). Obviously there may be a limitation for diagnostic application due to the reported elevation of anti-hsp70 levels in some autoimmune diseases such as systemic lupus erythematosis and rheumatoid arthritis (24,25). However, in autoimmune patients and also in the inactive TB cases, the antibodies appear to be predominantly cross-reactive with human hsp70. On the other hand it is encouraging that the anti-mycobacterial titres were approximately four times higher in the diagnostically difficult smear-negative and extrapulmonary cases as often as in smear-positive pulmonary TB. In fact, of the 11 sera with < 10 M/H ratio, 73% were

from patients with extrapulomanry TB. Hence, there would would be scope for improving further the mycobacterial specificity of the serology by using synthetic peptides or truncated recombinant segments (26) from the carboxy-terminal portion of the molecule which contains amino acid sequences with the most pronounced specificity for M.tuberculosis (12).

The mechanisms, which cause the apparently unique immunogenicity of hsp70 in the early stages of tuberculosis need further study. Proliferative responses of peripheral blood CD4 T cells to mycobacterial hsp70 were observed to be somewhat stronger than those to hsp65 (27) and CD8 T cell clones of hsp70 specificity were obtained from the peripheral blood of tuberculosis patients (28). The reported role of host hsp70 in processing of antigenic peptides (29) and expression on the surface of stimulated macrophages (30) could play a role for enhancing antibody responses to low levels of antigenic stimulation. The reported induction of synthesis of bacterial hsp70 together with other stress proteins during intracellular replication (31) could promote bacterial persistence via the ATP binding activity or by the special genetic regulatory elements (12) of hsp70. The pronounced serological response to hsp70 may indeed be signalling a distinct yet unrecognised function of this protein in the pathogenic host-parasite relationship.

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EXAMPLE 2

INTRODUCTION

The specificity of the immune repertoire in mycobacterial infections has been of interest from several aspects of the immunopathogenesis as well as for potentials towards diagnostic and vaccine development. Serological studies suggested that the antibody response in respect of species-specific protein antigens is restricted to relatively few constituents represented by the 38kDa, 19kDa and 14kDa antigens in tuberculosis and by the 35 kDa antigen

in leprosy (1). Antibody levels to all these antigens have been found most pronounced in patients with multibacillary forms of the disease which are clinically the most apparent and can be confirmed by bacteriological techniques. However, the sensitivity of detection of paucibacillary infection i.e. smear/culture negative tuberculosis using single or several antigenic probes has been less satisfactory (2-4).

A new important advancement in this direction has been achieved recently, by the demonstration that antibody levels to the mycobacterials hsp71 protein have been elevated to the same extent in patients with either smear-positive or smear-negative tuberculosis (5). The hsp71 antigen, originally identified by monoclonal antibodies is a stress protein constituent of mycobacterial (6-8). In view of this new opportunity for early serological diagnosis of paucibacillary disease it has been of further interest to identify those hsp71 epitopes which are both immunodominant as well as M.tuberculosis-specific. The need for focusing on the selection of species specific epitopes has been mandatory, to avoid the detection of cross-reactive antibodies to those parts of the hsp70 molecule which carries extensive structural homology with the corresponding proteins contained in all microbial and eukaryotic cells (7). Thus, we had chosen for epitope scanning the carboxy-terminal part of the molecule which has been found most polymorphic, in alignment of the hsp70 sequences from mycobacteria and other species(9).

MATERIALS AND METHODS

Peptides

The general net synthesis of the peptides purchased from Cambridge Research Biochemicals (CRB) (Cambridge, England) was comprised of 94 peptides based on 101 residues on the basis of the sequence of hsp71 of M. tuberculosis residues 509-621 (10). Non-cleavable octapeptides, overlapping by one residue starting at residue 509 (VRNQAETL) and ending at residue 621 (GRCPPRLG) were synthesised in solid-phase using the Geysen technique (11) on specially moulded, high-density polyethylene pins which are assembled into a plastic holder designed to hold 96 pins in the format and spacing of a microtitre plate. The tips of the pins have been chemically derivatised to provide functional groups to which amino acids may be coupled in an appropriate form in peptide synthesis. All peptides have received N-terminal acetylation. Positive and negative control peptide sequences were synthesised on the same block as the test peptides to give an indication of the sensitivity being achieved in the ELISA assay.

Sera

Selected sera which had previously been tested and all found as a high titre (1/2000-1/4000) to recombinant mycobacterial hsp70 (5) were collected from 2 cases of smear-positive pulmonary tuberculosis, 6 cases of smear-negative tuberculosis, and 1 case each of lepromatous leprosy, non-tuberculosis pulmonary disease of proven infection with M. avium, Crohn's disease and one healthy subject.

ELISA

This was performed following the recommended procedure from CRB. Reactions at the tips of the pins are carried out in polypropylene plates with wells in a standard 8 by 12 matrix matching the pin spacing. Briefly, the pins were precoated by dispensing 200 µl of pre-coat buffer (2% w/v bovine serum albumin), 0.1% v/v Tween 20,0.01 phosphate buffered saline pH 7.2) into each well of a microtitre plate. The pins were placed in the wells and incubated for 60 min at 20°C on a shaker at 100 rpm. Without washing, the pins were incubated overnight at 4°C in a microtitre plate containing 175 µl/well of human serum diluted 1/400 in pre-coat buffer. The pins were removed from the primary antibody preparation and washed four times 10 min each in a bath of 0.01 M PBS (pH 7.2) at room temperature with agitation. Then, the pins were placed in a microtitre plate containing 175 µl/well of peroxidase labelled goat anti-human IgG diluted 1/1000 in conjugated diluent (1% v/v sheep serum, 0.1% v/v Tween 20,0.1% w/v sodium caseinate and 0.01 M PBS pH 7.2) and incubated at 20°C for 60 min with agitation. The pins were washed as above and placed in a microtitre plate containing 150 µl/well of substrate solution, 0.5 mg/ml of 2,2'-azino-di[3-ethyl-benthiazoline sulfonate] in citrate buffer pH 4.0 and 0.01% w/v hydrogen peroxide. The reaction was stopped by removing the pins from the plate after 30 min and immediately the optical density was measured at 405 nm in a Titertek "Multiscan MCC II" spectrophotometer.

RESULTS

Altogether 13 sera were successively tested for binding to 94 overlapping peptides presented on a single 96-pin polyethylene block. There was no indication of any deterioration in the binding activities of peptides as judged by closely similar binding values to two pins containing control peptides. Optical density values from the ELISA assay of one representative serum from a patient with smear-negative pulmonary tuberculosis showed elevated antibody binding to several discretely separated clusters of adjacent peptides (figure 6). Four areas of peptides, based on sequences towards the carboxy-terminus and with the most consistent locallisation of increased binding had been designated as A (peptides 33-41), B (peptides 44-52), C (peptides 56-67) and D (peptides 74-85). Elevated binding values were also observed to peptids located further towards the amino-terminus; however, these presumably cross-reactive epitopes were omitted from evaluation. Peptide No. 77 showed low binding with all tested sera which we considered to be due to defective peptide structure or presentation in view of the consistent performance of adjacent peptides on both sides from peptide 77.

In order to evaluate the contribution of individual amino acids to the epitope structure, we have calculated the mean absorbance values for each residue from all 8 overlapping octapeptides containing the respective amino acid. This analysis was applicable to sequences, starting from the leucine at position 516, which represented the last

residue in peptide number 1. The results shown in Figure 7 demonstrate the values obtained from testing one serum each from patients with pulmonary tuberculosis (PT), calculated from values shown in figure 1), lung disease caused by M. avium (AM) and with lepromatous leprosy (LL). Antibody binding activities towards individual residues closely overlapped in their localisation and were clearly pronounced for epitopes B and D with each of the three tested sera. In contrast, binding to epitopes A and C identified with serum PT was not significant with the AM and LL sera. This individual variation in the binding patterns between sera supports the view that the four identified epitopes are of distinct specificity.

The core structures of each of the four identified epitopes have been alocated to six residues graded with the highest mean OD values. They are represented by sequences DAAVAE for epitope A, WRIGYF for epitope B, GEAGPG for epitope C and CVTGHW for epitope D (Figure 1). Alignment of these hexamers within the sequence of the hsp71 carboxy terminus from M. tuberculosis (10), with corresponding ssequences of M.leprae hsp70 (9), E.coli dnaK, (12) and of the human HSP70-1 gene (13) revealed, that the core of epitope A is overlapping in 5 out of the 6 core residues with the M.leprae sequence. In contrast, epitopes D, B and C shared only 2, 1 or no residue respectively with M.leprae. In view of the shared sequence of epitope A, it was surprising to find a lack of binding by the LL serum, selected with a high titre to several mycobacterial antigens. None of the four epitopes had significant

homology with either dnaK or human HSP70-1 structure. Predictive epitope evaluation of the carboxy-terminal sequence by the "Protean" programme (Proteus Biotechnology Ltd, Manchester, UK; kindly performed by J.Edwards at Peptide Technology Ltd. Sydney, Australia) allocated the highest score only to a single broad stretch of residues 565-591 which contains the epitope C (571-576) sequence.

A semi-quantitative evaluation of antibodies in the 13 tested sera against individual epitopes has been done by calculation of mean OD values from the 7 - 11 peptides, corresponding to each of the four epitopes. The results represented in Figure 8, demonstrate significantly higher antibody binding to each of the four epitopes in sera from tuberculosis patients when compared with non-tuberculosis controls. This difference was more significant for epitopes C and D ($p<0.001$) than for epitopes A and B ($p<0.01$). However, it should be remembered, that the serum from the healthy subject and the other 3 controls were purposely selected to contain high antibody binding to fulfil the topographic aims of the study. Hence, much better discrimination would be expected between TB patients and randomly selected non-tuberculosis patients and healthy controls.

DISCUSSION

The immunogenicity of protein antigens in terms of antibody formation is largely based on the recognition of epitopes displayed on native molecules by the immunoglobulin receptors of B cells (14). Indeed, the epitopes which react

with the bulk of antibodies in patients with tuberculosis are of conformational nature (1, 15, 16). Furthermore, the need for direct contact with B cells by the native antigens is corroborated by the fact that both 38kDa and 19kDa antigens are lipoproteins, secreted from live mycobacteria (17) and resulting in the highest antibody titres in multibacillary disease (2,3). In the paucibacillary stage of infection however, the presentation of those antigens of intracellularly replicating mycobacteria which had been processed within macrophages would stimulate an antibody repertoire of different specificity, directed predominantly to linear epitopes. This view is supported by the recent demonstration that antibody levels to the mycobacterial hsp71 antigen have been raised equally in sera from both bacteriologically positive and negative cases of tuberculosis (5). Similarly, anti-hsp70 antibodies were detected in the early stages of schistosomiasis (18). The pronounced immunogenicity of hsp71 during paucibacillary infection could be attributed to enhanced stress-induced synthesis during intracellular replication (19), or to surface expression (20,21) enabling recognition by the Ig receptors of B cells or merely to earlier priming by multiple cross-reacting commensal microbial organisms and autologous hsp70. Although the hsp71 protein is a predominantly intracellular constituent (8), its presence in media from short-term mycobacterial cultures (22) indicates some form of secretion from live bacteria which could be contributory to its immunogenicity for B cells.

In view of the extensive homology in the primary

structure of prokaryotic and eukaryotic hsp70 proteins (9), antibodies are likely to be produced against many cross-reactive epitopes of the hsp71 antigen. Indeed, elevated serum antibody levels to both microbial and human hsp71 have been found in a variety of infectious, chronic inflammatory and autoimmune diseases (5, 6, 23-26). However, it is notable, that antibodies produced following infection with Schistosoma mansoni were found to be predominantly directed against species-specific epitopes of hsp70 (29). The present peptide-scan analysis of the most polymorphic carboxy-terminal part of the hsp71 sequence identified four distinct linear epitopes. Localisation of these four epitopes was highly consistent with all 13 tested human sera without demonstrable microheterogeneity in the epitope structure which could have been revealed by antibodies selected from the broad human B cell repertoire.

Despite the limitation in the number of cycles in which the peptide-pins can be re-used, the analysis of a selected collection of sera allowed the unequivocal localiation of four core epitope sequences. Future bulk synthesis of these peptides will enable more extensive serological evaluation of the identified epitopes. It is possible, that the M.tuberculosis epitope aligned sequences from other microbial and eukaryotic species may also carry immunogenic epitopes. Their potential as species-specific probes for the involvement of hsp70 could be valuable in the study of immunopathogenesis of various infectious diseases or autoimmunity and possibly for immunodiagnostic applications.

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Claims

1. A peptide comprising a sequence the same as or immunologically equivalent to a linear epitope of the carboxy terminal region of Micobacteria tuberculosis Hsp71 or a corresponding heat shock protein or any other mycobacterial pathogen.

2. A peptide according to claim 1 comprising a sequence the same as or immunologically equivalent to a linear epitope of the carboxy terminal of M.tuberculosis Hsp71.

3. A peptide according to claim 1 containing a sequence comprising at least five residues of one of the following linear epitope core sequences:

DAAVAE

WRIGYF

GEAGPG

CVTGHW

4. A peptide according to claim 3 containing a sequence:

DAAVAE

WRIGYF

GEAGPG

CVTGHW

5. A peptide according to claim 4 containing one of the following sequences:

KVDAAVAE

VDAAVAEA

DAAVAEAE

GTWRIGYF

TWRIGYFG

WRIGYFGH

GDGEAGPG

DGEAGPGV

GEAGPGVA

SGCVTGHW

GCVTGHWR

CVTGHWRC

6. A peptide according to claim 1 or claim 2 containing a sequence immunologically equivalent to a sequence according to any one of claims 3 to 5.

7. A peptide according to any preceding claim bound to a solid carrier or a detectable label.

8. A peptide according to claim 7 bound to an enzyme label, cromophore, fluorophore or radio-isotope.

9. A peptide according to any preceding claim for use in a diagnostic process practised on the human or animal body.

10. A diagnostic test process comprising contacting a peptide according to any one of the preceding claims with a body fuid sample sample taken from an individual suspected of having a microbacterial infection.

11. A diagnostic test ket comprising a peptide according to any one of claims 1 to 9 optionally bearing a label or linked to a solid support and optionally also comprising buffer, positive or negative control materials, labelling reagents, reagents for detecting a label and other conventional components.

Fig.1.

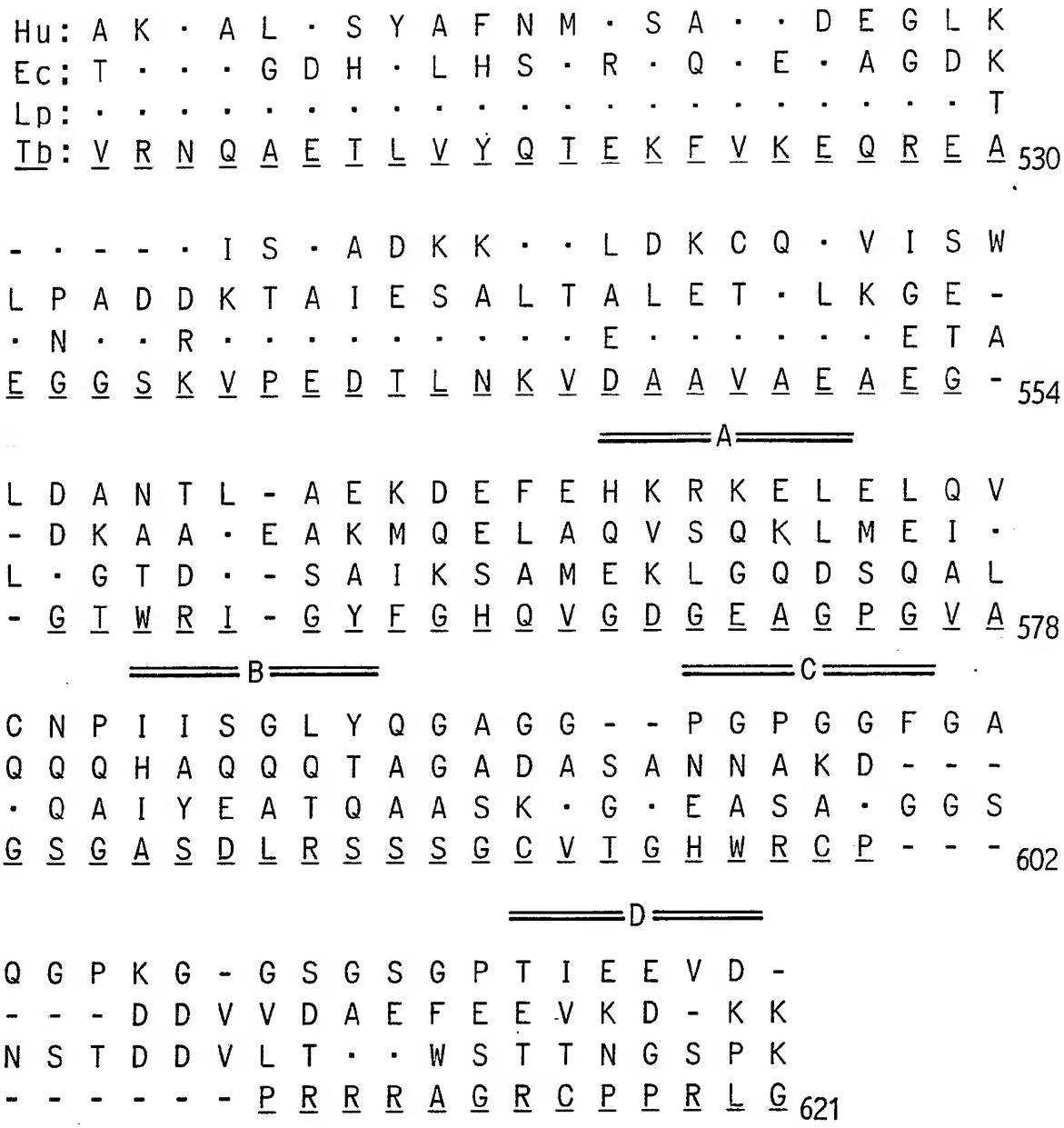
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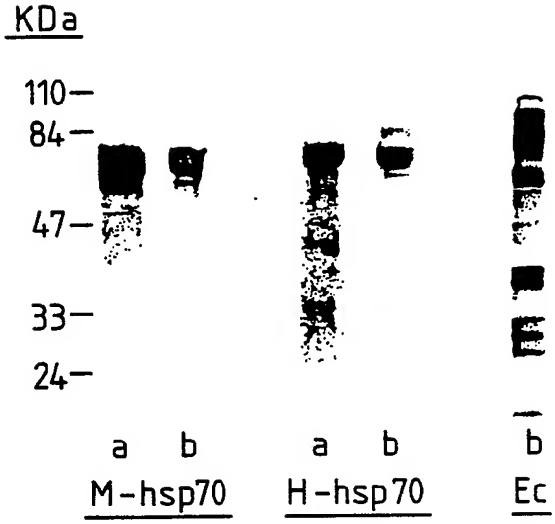
Fig. 2.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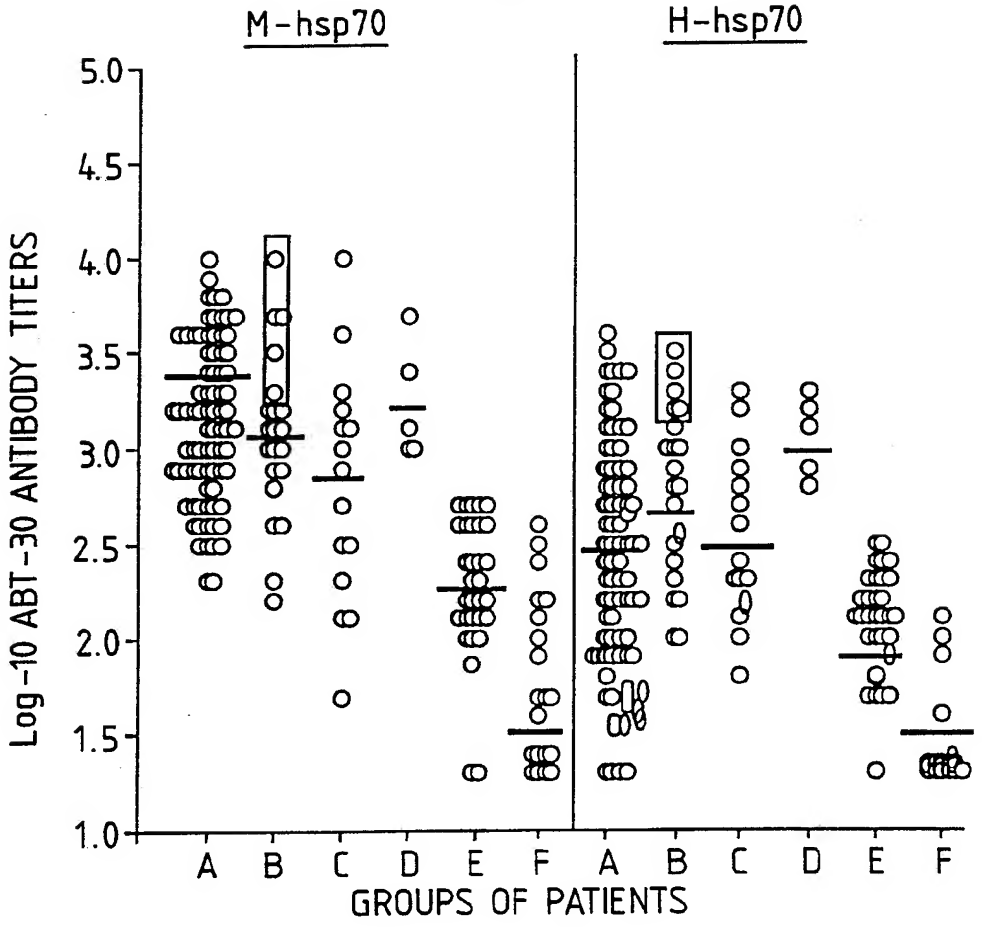
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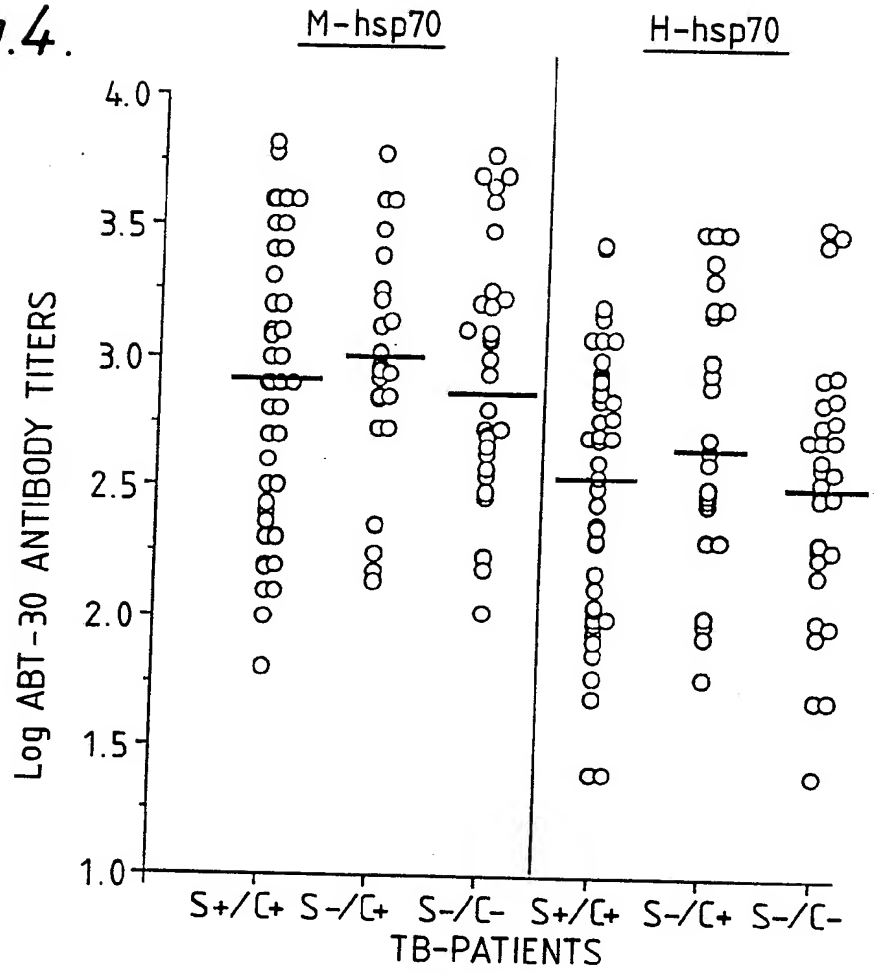
Fig.4.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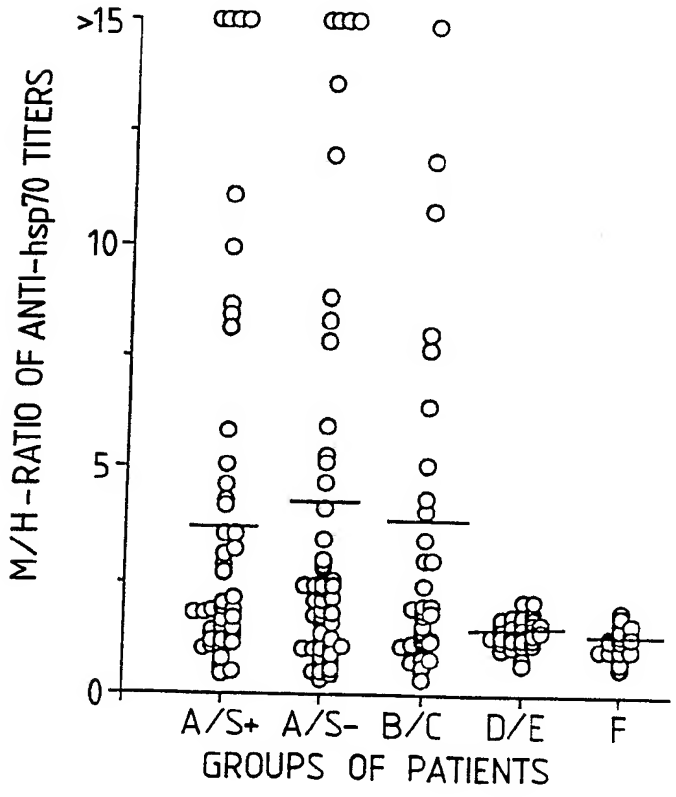
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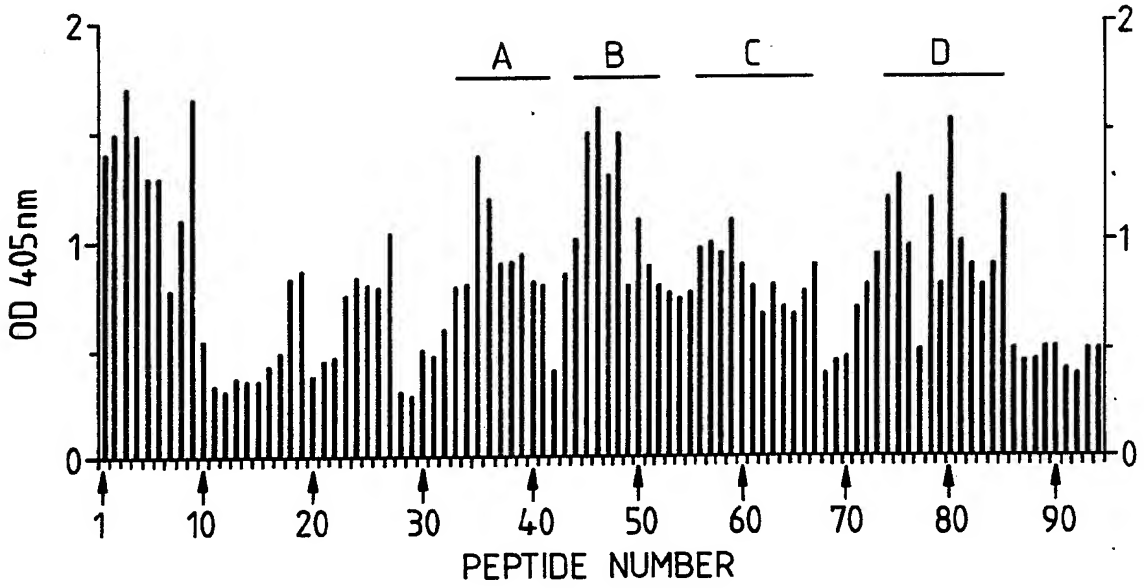
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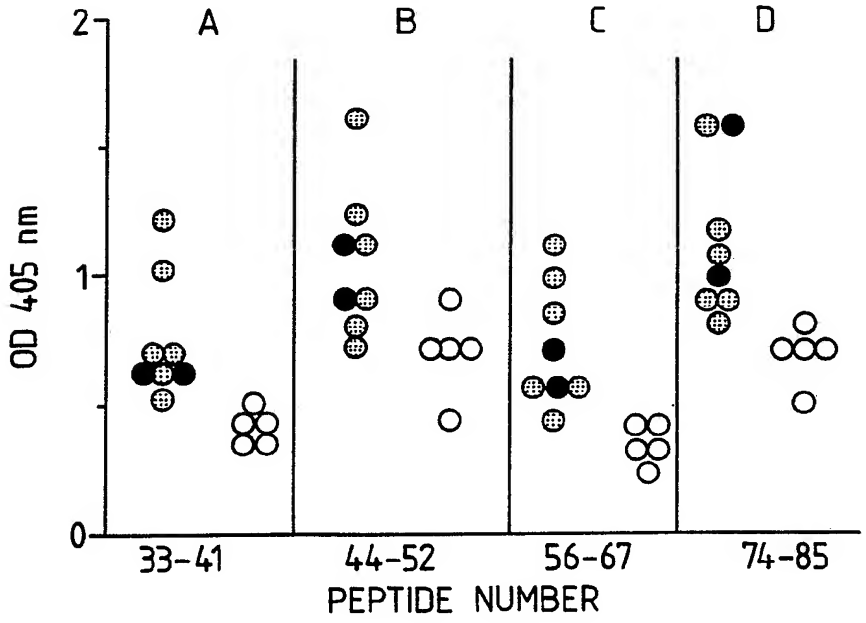
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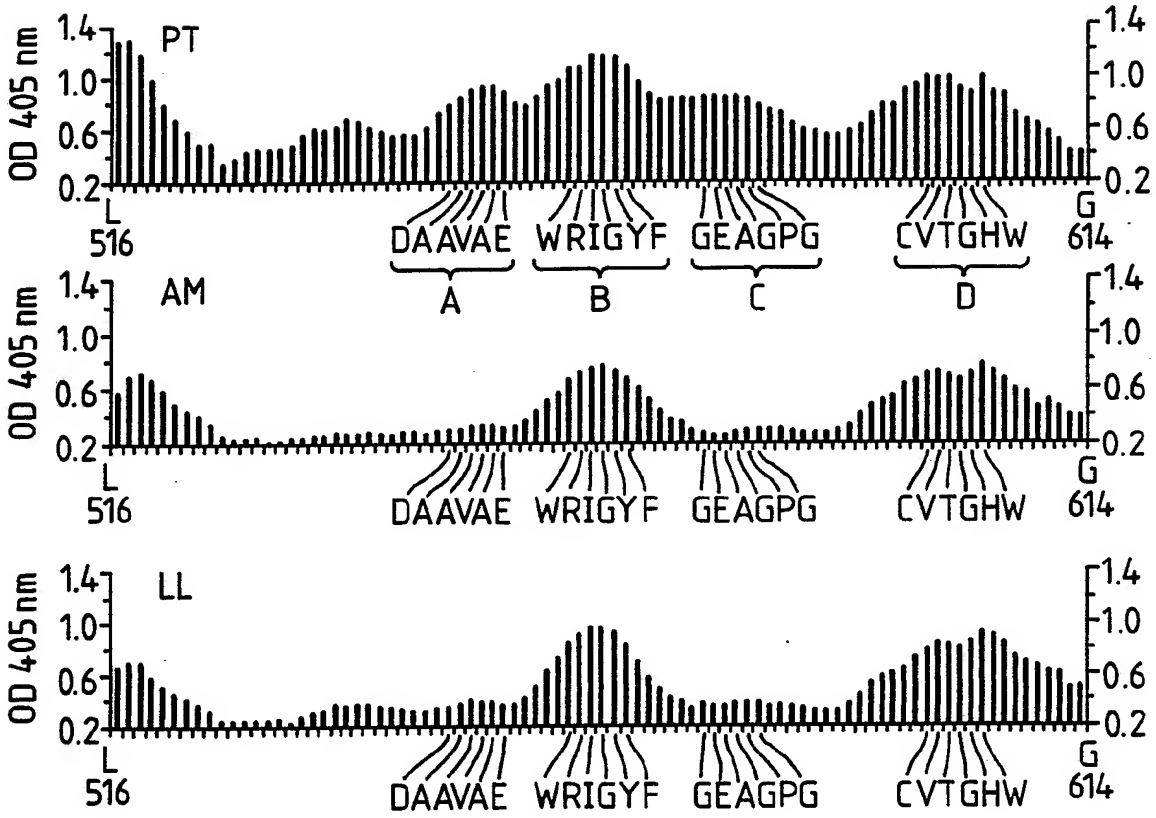
Fig. 7.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INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00087

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/06; C07K7/08; C07K7/10; G01N33/569

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	IMMUNOLOGY AND INFECTIOUS DISEASES vol. 1, no. 5, December 1991, pages 323 - 328 A.A.F. ELSAGHIER ET AL. 'ELEVATED ANTIBODY LEVELS TO STRESS PROTEIN HSP70 IN SMEAR-NEGATIVE TUBERCULOSIS' cited in the application see page 324, left column, paragraph 2 - right column, paragraph 2 see page 327, left column, paragraph 2 --- -/--	1-11

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 MAY 1993	
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	FUHR C.K.B.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 85, June 1988, WASHINGTON US pages 4267 - 4270 D. YOUNG ET AL. 'Stress proteins are immune targets in leprosy and tuberculosis' see page 4267, right column, last paragraph - page 4268, left column, paragraph 2; figure 1	1-6
P,X	MOLECULAR IMMUNOLOGY vol. 29, no. 9, September 1992, pages 1153 - 1156 A. ELASGHIER ET AL. 'Localisation of linear epitopes at the carboxy-terminal end of the mycobacterial 71 kDA heat shock protein' see the whole document	1-11
X	WO,A,8 805 823 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 11 August 1988 see claims 10,11,13,14,23-27; figure 7	1-6,9-11
A	GB,A,2 239 246 (AKTIEBOLAGET ASTRA) 26 June 1991 see chapter 5.3-5.3.4 on pages 16-19 see claims 1,2,8,13,17,19	1-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9300087
SA 68962

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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		EP-A- 0345299	13-12-89
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		EP-A- 0479971	15-04-92
		WO-A- 9114448	03-10-91